Confined Study of a Transgenic Pink Bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

Environmental Assessment

(October 1, 2001)

Finding of No Significant Impact
Response to Comments
Environmental Assessment

Finding of No Significant Impact
for
Confined Study of a Transgenic Pink Bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

The U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), is proposing to issue a permit for a confined field study of a transgenic pink bollworm (PBW), *Pectinophora gossypiella.* In support of our permitting decision, we have prepared this environmental assessment (EA) under APHIS regulations at 7 CFR Parts 340 and 372 and the National Environmental Policy Act, 42 U.S.C. 4321. The permit applicant is the USDA, APHIS, PPQ Phoenix Plant Protection Center in Phoenix, Arizona and the application number is 01-029-01r.

The PBW was described from larvae recovered from infested cotton bolls in India in 1843. It has since become one of the most destructive pests of cotton in many of the major cotton-growing regions of the world and may be the most destructive pest of cotton worldwide. Pink bollworm larvae feed inside the growing cotton boll, destroying the cotton. Costs relating to prevention, control, and yield losses have been estimated by the National Cotton Council of America to be more than $24 million annually. In Egypt, China, and Brazil, it commonly causes cotton losses of up to 20 %, although losses can be much higher.

This insect is now a pest in Texas, New Mexico, Arizona, and California. It has also occurred in Oklahoma, Arkansas, Mississippi, Missouri, Tennessee, Louisiana, and in southern Florida. It prefers cotton, but will feed on okra, kenaf, and hibiscus. The USDA, APHIS assists States in controlling the pest and preventing its spread to other States. APHIS enforces a quarantine in infested areas, requiring certification for the interstate movement of regulated articles.

Alternatives we considered in this EA are No Action or Issuance of the Permit. Under the No Action Alternative, APHIS would not issue the permit as described in the EA and the confined field study would not take place. Under the Issuance of the Permit alternative, APHIS would issue a permit for the confined field study and allow the research for the purpose of determining if the green fluorescent genetic marker affects the behavior and performance of the PBW to proceed.

Based on the analysis carried out in the EA, APHIS has reached a finding of no significant impact (FONSI) to the environment and intends to issue a permit for the confined field study of a transgenic PBW. Before reaching this decision, APHIS requested, received, and considered comments on the EA from the public. A response to these comments is included as an attachment to this FONSI statement. The reasons for the decision are:

- The possibility of the genetically modified organism reverting to or undergoing some form of unanticipated genetic transformation is exceedingly low. The proposed highly confined field tests are designed to prevent reproduction and to prevent any unexpected traits from being transmitted to offspring.
- It is highly unlikely that the enhanced green fluorescent protein (*EGFP*) gene would persist in the environment because it provides no fitness advantage to the PBW. Even if the *EGFP* gene were present in a field population of the pink bollworm, it would not confer a selective advantage and the insects would likely lose the gene and revert to wild-type insects over time.
- Multiple levels of physical and biological confinement in the proposed research are designed to contain the transgenic PBW. These containment measures are: (1) isolation from other cotton fields by distance; (2) isolation of the insects from the environment by screen cages; (3) reproductive sterilization; (4) removing wings of females and placing them in secondary cages; (5) male pheromone traps; (6) destruction of the cotton that may contain bollworms; (7) treating the area with a high ratio of sterilized bollworms; and (8) insecticide treatment, if required.
- The PBW is not native to the United States and there are no known sexually compatible species in North America.
- There is no current evidence that this gene can be transferred through predation, natural decay, or parasitism. Parasitic and infectious organisms are unrelated to the PBW and would not be expected to assimilate functional DNA from their hosts leading to modification of the parasite or microorganism.
- The confined research would not result in an additional pesticide load on the environment.
- The research would not disproportionately affect minority or low income populations, or disproportionately affect children, or result in any environmental health risks or safety risks to children.
- APHIS has determined that, based on the location of the test field and the measures designed to contain the transgenic PBW, the proposed test will have no effect on listed threatened, endangered, or candidate species.

The effects on environmental quality are expected to be nonexistent or very low because the *EGFP* marker gene is not known or expected to negatively impact the environment. Additionally, the experiment is biologically and physically highly confined. Therefore, the degree of uncertainty is very low.

Signed by:
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Assistant Director
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Date signed:
October 1, 2001

Attachment
Finding of No Significant Impact
Response to Comments
APHIS Permit No. 01-029-01r

In response to a notice published in the *Federal Register* on June 21, 2001 (66 FR 33226, Docket no. 01-024-1), the Animal and Plant Health Inspection Service (APHIS) received 9 comments on the environmental assessment (EA) prepared for permit application number 01-029-01r during the designated 30-day comment period which ended July 23, 2001. Comments were received from universities, environmental and consumer groups, a university medical research center, a crop protection association, a cotton industry organization, and a cotton growers' group. Four comments were in favor of approval of the field test permit application, and three were opposed. Because three of the comments critical of the proposed field test were written by the same commenter and were identical in content, they have been considered as a single comment. We have confined our response to comments of direct relevance to the issuance of a permit for the subject confined field test.

The commenters favoring issuance of a permit for the subject field test stressed the thoroughness of the planned containment measures and safeguards, the negligible risk, the adequacy of the EA, and the need for gathering data for use in pink bollworm (PBW) control. Among the issues raised by commenters critical of the proposed PBW field test were the desirability of additional data on transgene stability, the need for an independent assessment of the permit application, the adequacy of the proposed containment measures, potential human health risks, and alleged deficiencies in APHIS' compliance with the requirements of certain Federal laws, including the National Environmental Policy Act (NEPA) and the Endangered Species Act (ESA).

The three commenters expressing concerns about the proposed field test called for additional testing of the transgene used to transform the subject PBW to provide data on stability and related subjects. APHIS welcomes testing and data on any issues relating to the subject field test. However, questions concerning transgene stability in the PBW expressing enhanced green fluorescent protein (EGFP) were satisfactorily addressed early in the development of the transgenic PBW, and in the multiple generations of transgenic PBWs reared in the Phoenix facility with no evidence of instability. The details are provided in the subject permit application and Appendix III of the EA, "Development of a Genetically Engineered Pink Bollworm." Should experiments be conducted to provide additional data, they would not provide information relevant to a fully confined study in which multiple levels of biological and physical containment preclude virtually any contact between the transgenic PBW and the environment.

Two commenters contended that APHIS should have assembled an independent team to evaluate the subject permit application and any potential risks presented by the proposed field test. In fact, the subject limited field test is the result of several years of consultations among APHIS arthropod experts in headquarters and in the regions, the university researchers responsible for development of the transgenic PBW, and State agriculture officials. The safeguards outlined in the permit application are a result of these multiple consultations.

While recognizing the limited implications of the subject field test, two commenters addressed the so-called "larger picture" associated with the application of genetic engineering technology to arthropod research. One commenter further proposed that a full Environmental Impact Statement (EIS) be prepared for the entire transgenic PBW Sterile Insect Technique (SIT) program. These comments presume that APHIS is engaged in a transgenic arthropod research program, while APHIS is not a research agency and such a program has not been undertaken. The PBW SIT program is a cooperative one involving the private sector, State officials, and APHIS. The use of genetic engineering technology to insert a marker in PBW is simply another means of identifying the sterile insects used in the control program. Should a transgenic arthropod development program be contemplated, or additional field tests conducted, the appropriate environmental documents will be prepared in advance for public comment. APHIS does expect, however, to undertake an exploration of the issues and impacts associated with the release of certain transgenic arthropods through the NEPA EIS process.

Two commenters claimed that the planned confinement measures, namely the field cage study, would not contain the possible interaction of certain insect viruses, e.g., baculoviruses, and the transgenic PBW. Such interaction is highly unlikely because these viruses are host specific and few infect PBW, the viruses are fragile and will be adversely affected by the strong Phoenix sun, and only adult PBW will be used in the test and adults are not normally susceptible to infection by such viruses. Further, the numerous confinement measures proposed include specific provisions which preclude the interaction of insect viruses and PBW. The same commenter expressed concern that baculoviruses are a possible source of human and mammalian infectivity through the rescue of the inactivated *piggBac* transposon in the transgenic PBW by baculoviruses. However, baculoviruses are highly specific to a few species of insects for each virus. And while laboratory experimentation appeared to support the possibility that baculoviruses might be used for gene therapy in mammalian cells, later work shows that baculoviruses are not infectious in mammalian cells. The potential of a very selective insect virus somehow becoming a human pathogen, or even a non-pathogen gaining access to human cells, or cells of any vertebrate or of the virus carrying genetic material from an insect to a vertebrate has never been demonstrated, nor has that been a basis upon which to design an experiment to test any hypothesis (Miller and Peloquin).

One commenter provided an extensive critique of the structure of the EA prepared for the subject field test, citing alleged deficiencies and nonconformance with the provisions of NEPA. APHIS has reformatted the EA for readibility, but the content of the assessment is basically the same as that contained in the document presented for public comment. In response to the commenter, we note that fully contained field tests of genetically engineered organisms considered regulated articles under 7 CFR part 340 may be categorically excluded under the APHIS NEPA Implementing Procedures at 7 CFR part 372 when the "means through which adverse environmental impacts may be avoided or minimized have actually been built right into the actions themselves " (7 CFR.§ 372.5(c)). Only in a most limited sense does the subject biologically and physically contained field trial of a sterile insect bearing a marker gene require

an EA as an exception to the categorically excluded actions as defined in 7 CFR § 372.5(d)(4). This commenter also contended that APHIS had failed to describe the affected environment in which the proposed field test is to be conducted. On the contrary, in addition to the description of the field test site in the permit application, which is incorporated in the EA, the EA provides a description of the Maricopa County, AZ area which is more than adequate for assessing any potential impacts of the proposed field test. An additional comment from the same source contended that APHIS had failed to consult with the U.S. Fish and Wildlife Service (FWS), as required under Section 7 of the ESA concerning the proposed transgenic PBW field test. In fact, APHIS notified FWS in writing in January 2001 of the proposed field trial, whose safeguards and site preclude any contact with endangered or candidate species protected by the provisions of the ESA.

Confined Study of a Transgenic Pink Bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

Environmental Assessment

(October 1, 2001)

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I. PURPOSE and NEED

This Environmental Assessment (EA) was prepared to assess any potential adverse environmental effects of a confined field study of a particular strain of the transgenic pink bollworm (PBW), *Pectinophora gossypiella.* The application for a permit was submitted January 17, 2001 by the U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Plant Protection Center, Phoenix, Arizona. The application number is 01-029-01r and it is located at: http://www.aphis.usda.gov/biotech/arthropod/

A. Introduction

The PBW was described from larvae recovered from infested cotton bolls in India in 1843. It has since become one of the most destructive pests of cotton in many of the major cotton-growing regions of the world and may be the most destructive pest of cotton worldwide. The first reported cotton infestation in North America occurred in 1911 in Mexico, presumably from Egyptian cotton seed shipments (Nobel, 1969). In the United States, the PBW was detected first in Robertson County, Texas in 1917 (Scholl, 1919). By 1926, the pest had spread from Texas through New Mexico and into eastern Arizona, and it became a major economic pest of cotton in Arizona, southern California, and northwestern Mexico by 1965 (Burrows et al., 1982). This insect is now a pest in Texas, New Mexico, Arizona, and California. It has also occurred in Oklahoma, Arkansas, Mississippi, Missouri, Tennessee, Louisiana, and in southern Florida. It prefers cotton, but will feed on okra, kenaf, and hibiscus. The USDA, APHIS assists States in controlling the pest and preventing its spread to other States. APHIS enforces a quarantine in infested areas, requiring certification for the interstate movement of regulated articles. From: October 1995, USDA, APHIS, Plant Protection and Quarantine. URL at: http://www.aphis.usda.gov/oa/pubs/fspbw.htm

The San Joaquin Valley of California remains the last cotton growing area in the Southwest that is not infested with PBW. Prevention of its establishment in this valley is attributed primarily to the ongoing Sterile-Insect Technique (SIT) program established jointly in 1968 by USDA, APHIS, California Department of Food and Agriculture, and the California cotton growers. The SIT program for the PBW is described in Appendix I.

Pink bollworm larvae feed inside the growing cotton boll, destroying the cotton. Costs relating to prevention, control, and yield losses have been estimated by the National Cotton Council of America to be more than $24 million annually. In Egypt, China, and Brazil, it commonly causes cotton losses of up to 20 %, although losses can be much higher.

The PBW has four stages of development: egg, larva, pupa, and adult. In early June, female moths lay 100 to 200 eggs on young cotton bolls. The eggs hatch in about five days and develop into larvae, the stage that damages cotton. The fully-grown larvae are 7 to 10 mm long (1/4 to 3/8 inch). First and second instar larval bodies are smaller, ivory in color, and have dark heads. Late instar larvae have bodies with pink bands. Adult moths are grayish brown and about the same length. Their wingspan is 15 to 20 mm (5/8 to 7/8 inch). Larvae bore into the cotton bolls and feed from 10 to 14 days on the seed.

One larva eats a whole seed or parts of several seeds. When larvae finish feeding, they either drop to the ground or remain in the boll to pupate. Pupation can also take place under ground trash. Pupae emerge as moths in 8 to 10 days. The female start laying eggs 1 to 3 days later after mating. Adults are active only at night and live about 10 days. In warmer areas, most larvae overwinter in cotton or okra pods left in the field after harvest. In colder climates, larvae may form cocoons in the soil for overwintering. Larvae can also remain in cotton seed after the cotton is ginned, and if the seed is not fumigated, some of the larvae can emerge from the stored seed the next spring. The PBW is well adapted to the long growing seasons prevalent in the desert valleys of the southwest where 5 to 6 generations develop each year. Egg-to-adult development takes 26–32 days during the cotton-growing season. Integrated pest management of the PBW is discussed in Appendix II.

B. Purpose and Need

APHIS has two levels of need regarding transgenic pink bollworms. One is the Agency's need to decide about issuing the permit and whether or not there would be any undesirable consequences. The second need is for the Agency to do the research to evaluate the biological performance in caged field tests of a strain of the PBW genetically engineered to express an enhanced green fluorescent protein (*EGFP*) marker gene derived from a jellyfish. In this EA we address only the need regarding issuing the permit.

Before genetically transformed insects expressing green fluorescence as a marker can be considered as an improved means of monitoring the effectiveness of the SIT program, there is a need to know more about their behavior and performance under field conditions. The purpose of the caged field tests is to determine field fitness of the EGFP strain of PBW and compare its performance to its mass-reared nontransgenic counterparts. Genetically marked insects can be distinguished from a native pink bollworm by screening with a fluorescent microscope and/or PCR (Peloquin and Miller, 2000). This testing requires field research that would be fully contained and protected against vandalism. Technical aspects of the development of the genetically engineered PBW expressing green fluorescence are discussed in Appendix III.

The multiple levels of physical and biological confinement are: (1) isolation from other cotton fields by distance; (2) isolation of the insects from the environment by screen cages; (3) reproductive sterilization; (4) removing wings of females and placing them in secondary cages; (5) male pheromone traps; (6) destruction of the cotton that may contain bollworms; (7) flooding the area with a high-ratio of sterilized bollworms; and (8) insecticide treatment, if required.

The stability of the introduced gene was demonstrated by rearing at the APHIS Phoenix Plant Protection quarantine facility of 20 generations of the transgenic PBW with no evidence of instability. The only discernable difference found in the biology of the EGFP strain PBW, when compared to its non-genetically modified parental strain, was that the EGFP female moths produced 19.8% fewer eggs than non-transformed PBW and their

successful egg hatch rate was 26% lower.

C. Regulatory Authority

The United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) regulations under 7 CFR Part 340, Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests, were set forth under authority granted by the Plant Protection Act (7 U.S.C. 7701-7772). These regulate certain genetically engineered organisms and products. A genetically engineered organism is considered a regulated article if the donor organism, recipient organism, vector or vector agent used in engineering the organism belongs to one of the taxonomic groups listed in the regulation and is also a plant pest, or if there is a reason to believe it is a plant pest. The pink bollworm, *Pectinophora gossypiella* (Saunders), is the recipient organism and is a plant pest. The donor source of the transposon, *piggyBac*, is the cabbage looper, *Trichoplusia ni* (Huebner), which is also a plant pest. A transposon or transposable element is a segment of a gene which is able to move from one chromosome site to another site in the same cell and may cause other genetic material to move with it. They are used in molecular biology to help insert new genes into organisms.

The authority for 7 CFR Part 372, National Environmental Policy Act (NEPA) Implementing Procedures, is 42 U.S.C. 4321. Under 7 CFR § 372.5, Classification of actions, (c) Categorically excluded actions include (ii) Permitting, or acknowledgment of notifications for, confined field testing of genetically engineered organisms and products, except (4) when a confined field test of genetically modified organisms or products involves new species or organisms or novel modifications that raise new issues. A genetically modified PBW field trial outside, even though it is contained, may meet this exception to a categorical exclusion and be subject to NEPA because it raises new issues of public concern that should be addressed in an environmental assessment.

D. Relevant Issues

1. The possibility that the genetically modified organism will undergo some form of unanticipated genetic transformation.
2. Persistence compared to wild-type.
3. Physical and biological confinement of the genetically modified PBW.
4. Gene transfer to related species.
5. Gene transfer to predators, saprophytres, or parasites.
6. Potential impacts on humans, including minorities, low income populations, and children.
7. Effects on chemical (pesticide, herbicide, fungicide) load on the environment.
8. Risks to nontarget plants and animals including threatened and endangered species.

II. ALTERNATIVES

A. No Action

APHIS permit not issued. This alternative is comparable to a no action alternative.

B. Issuance of the Permit

Issuing this permit would allow the research to proceed for the purpose of determining if the green fluorescent genetic marker affects the behavior and performance of the PBW. Biological fitness data will be obtained on the genetically modified strain of PBW.

The application for a permit to conduct the confined field trials of the EGFP strain of PBW was submitted by the USDA, APHIS, Phoenix Plant Protection Laboratory, Phoenix, AZ. Transgenic insects reared in this facility will be confined in screen cages (3.6 by 7.3 by 1.8 m) placed over cotton in the field. The structure of the field cages consist of a 2.54-cm galvanized pipe frame covered with a 16 x 16 mesh (256 openings per square inch) fiberglass screen with reinforced corners to prevent tears. The cage also has a plastic skirt 30.5 cm in width along the bottom, which is buried in the soil to prevent moth escapes. The selected cotton field is located in a nonresidential urban area in southeastern Phoenix. No other cotton fields are within three miles of this field. Although there are a few ornamental hibiscus around residences within a mile of the testing site, the permit applicants have found PBW will not complete its biological development on the contemporary ornamental cultivars locally available from nurseries and other area retailers.

All insects in the study will be irradiated with 20 kilorad (kr) of $Cobalt_{60}$ prior to the study to insure sterility. This treatment produces virtually 100% sterility. A previous study by Miller et al. (1984) indicated under laboratory conditions, that a population of 720,000 PBW produced one normal adult per 1,000 parent females. Under field conditions, no F_1 progeny were produced from a study of 2.25 million PBW in field cages. Only male moths of the genetically modified strain of PBW would have the potential to escape into the environment since females are double caged and also have their wings clipped. However, in the unlikelihood that any males escape and mate with an unmodified or wild-type female, she would not produce offspring from that male because he has been sterilized by radiation.

All test females will have their wings clipped so they cannot fly. They will also be contained inside the primary screen cages in paper 1-gallon bucket secondary cages with an open top and a barrier of grease to prevent the non-flying females from crawling out of the containers.

Pheromone traps will be placed in a grid pattern in the field surrounding the cages to capture any males that might escape the cage. If a catastrophic event should occur to the field cages, all fruiting forms under the cages will be collected and destroyed and the field will be treated with a high-ratio of sterilized PBW. Insecticides may also be used as

a final measure of control, if required.

Transgenic PBW that are no longer needed will be disposed of by freezing at –20° C for 24 hours. This will destroy all life stages of this insect. Transgenic PBW recaptured in the field trails will be disposed of by freezing at –20° C for 24 hours. All plant fruiting forms in the test cages will be disposed of by freezing at –20° C for 24 hours when the study is completed. This will destroy life stages that may infest the fruiting forms.

A description of the individual experiments that are planned may be found in Appendix IV.

III. AFFECTED ENVIRONMENT

The experiments will be conducted in field cages located in a cotton field in Maricopa County, Arizona. The selected cotton field is about three acres in size and located in a nonresidential urban light industrial area in southeastern Phoenix. No other cotton fields are within three miles of this field. Although there are a few ornamental hibiscus around residences within a mile of the testing site, the permit applicants have found that the PBW will not complete its biological development on the contemporary ornamental cultivars available in the area from nurseries and other retailers. The test site is within the geographic area that has already been infested by pink bollworms. The proposed experiments would be conducted under high security containment and be guarded against vandalism.

IV. ENVIRONMENTAL CONSEQUENCES

Summary of Consequences

Issues	No Action	Issue Permit
The possibility that the genetically modified organism will undergo some form of unanticipated genetic transformation	No effect	No effect
Persistence compared to wild-type	No effect	No effect
Physical and biological confinement of the genetically modified PBW	No effect	No effect
Gene transfer to related species	No effect	No effect
Gene transfer to predators, saprophytes, or parasites	No effect	No effect
Potential impacts on humans, including low income populations and children	No effect	No effect
Effects on chemical (pesticide, herbicide, fungicide) load on the environment	No effect	No effect

Issues	No Action	Issue Permit
Risks to nontarget plants and animals including threatened and endangered species	No effect	No effect

A. Deny the Permit Application.
To deny the permit application would have no expected potential adverse environmental impacts and would prevent this contained field research from proceeding.

B. Issuance of the Permit.
The proposed action is not expected to have any adverse environmental impacts for the following biological and physical reasons:

No adverse consequences to nontarget organisms or environmental quality are expected from incorporation of this marker into the pink bollworm. The unmodified pink bollworm has no *EGFP* gene; therefore, it does not fluoresce green when illuminated. Neither *piggyBac* transposase replication activity, nor any antibiotic resistance is conferred to the transgenic PBW by the introduced genetic material as genetic material encoding these proteins was not integrated into the PBW genome.

1. Possibility that the genetically modified organism will undergo some form of unanticipated genetic transformation that may effect the environment
The possibility of the genetically modified organism reverting to or undergoing some form of unanticipated genetic transformation are low based on a 20-generation study, and in addition, the proposed highly confined field tests are designed to prevent reproduction and to prevent any unexpected traits from being transmitted to offspring.

2. Persistence compared to wild-type
It is highly unlikely that the *EGFP* gene would persist in the environment because it provides no fitness advantage to the PBW. In fact, a 20-generation study of EGFP PBW showed that there was a loss of fitness evidenced in the female's ability to produce eggs and egg survivability was also reduced in the EGFP strain. The enhanced EGFP female moths produced 19.8% fewer eggs than non-transformed PBW and their successful egg hatch rate was 26% lower (Miller et al., 2001). Even if the *EGFP* gene were present in a field population of the pink bollworm, it would not confer a selective advantage and the insects would likely lose the gene and revert to wild-type insects over time.

3. Physical and biological confinement
The multiple levels of physical and biological confinement in the proposed research are: (1) isolation from other cotton fields by distance; (2) isolation of the insects from the environment by screen cages; (3) reproductive sterilization; (4) removing wings of females and placing them in secondary cages; (5) male pheromone traps; (6) destruction of the cotton that may contain bollworms; (7)

treating the area with a high ratio of sterilized bollworms; and (8) insecticide treatment, if required.

4. Gene transfer to related species
The PBW is not native to the United States and there are no known sexually compatible species in North America.

5. Gene transfer to predators, saprophytes, or parasites
Pink bollworms may be eaten by predatory insects, birds, or mammals that venture into cotton fields. However, only the adult stage will be included in the study which further reduces any chance of predation or parasitism since larvae are typically preferred over adults. The green fluorescent protein is a naturally occurring protein, not known to cause adverse effects. The gene has been found in nature only in the jellyfish from which it is derived. Jellyfish have been prey or subject to saprophytic digestion by other organisms since their origin. There is no current evidence that this gene has been transferred through predation, natural decay, or parasitism. The normal digestive process of predators would preclude transfer of functional genetic material to the predator and this phenomenon is not expected with insectivores. Pink bollworms may also serve as hosts for parasitic insects, nematodes, and various microorganisms. These parasitic and infectious organisms are unrelated to the PBW and would not be expected to assimilate functional DNA from their hosts leading to modification of the parasite or microorganism.

6. Potential impacts on humans, including minorities, low income populations, and children
These requirements are specified in Executive Orders 13045 and 12898 and address the identification of health or safety risks that might disproportionately affect children or have adverse impacts on minorities and low income populations. The proposed actions are not expected to adversely affect any of these groups. Lepidoptera have also been specifically excluded from consideration as human health hazards by a working group of the American Society of Tropical Medicine and Hygiene on biosafety of transgenic arthropods.

7. Effects on chemical (pesticide, herbicide, fungicide) load on the environment
US Environmental Protection Agency registered pesticides, primarily insecticides, are used more intensively on cotton than most other crops. The confined research would not result in an additional pesticide load on the environment.

8. Risks to nontarget plants and animals including threatened and endangered species
APHIS has determined that, based on the location of the test field and the measures designed to contain the transgenic PBW and the fact that there are no threatened and endangered species in the area, the proposed test will have no effect on listed threatened, endangered, or candidate species.

C. Likelihood of controversy of effects on environmental quality
The effects on environmental quality are expected to be nonexistant or low because the *EGFP* marker gene is not known or expected to negatively impact the environment. Additionally, the experiment is biologically and physically contained. However, some controversy is expected because the experiments involve a genetically modified insect and many people fear or dislike insects and other arthropods such as spiders. This experiment was specifically suggested by a working group sponsored by the Food and Agriculture Organization of the United Nations as a first experiment using transgenic arthropods (Ashburner et al. 1998).

D. Degree of possible uncertain effects on the environment and unique or unknown risks
The experiments are biologically and physically highly confined and the *EGFP* gene is not known or expected to affect the environment, therefore, the degree of uncertainty is low. Furthermore, Miller et al. (2001) have shown that the *EGFP* gene provides no selective advantage over 20 generations of the PBW.

E. Consistency of proposal with other environmental requirements
The proposal is believed to be consistent with other environmental requirements.

V. PREPARATION, CONSULTATION, AND REVIEW

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APPENDIX I

Pink bollworm Sterile-Insect Technique (SIT) Program

The objective of the sterile-insect technique (SIT) program is to prevent pink bollworm (PBW) moths blown into the San Joaquin Valley of California on storm systems originating in Mexico

and the Southern California cotton growing regions from establishing infestations (Staten et al. 1992). The program operates by placing radiation sterilized moths on cotton in the San Joaquin Valley. Sterile insects mate with insects in the field and do not produce offspring. The San Joaquin Valley of California remains the last cotton growing area in the Southwest that is not generally infested with PBW. Prevention of its establishment in this valley is attributed primarily to the ongoing SIT program established in 1968 jointly by APHIS, California Department of Food and Agriculture, and the California cotton growers (Miller et al.1984). All sterile PBW used in this project are mass-reared in a United States Department of Agriculture, Animal and Plant Health Inspection Service facilities in Phoenix, Arizona.

For the sterile insect method to succeed, a ratio of 60 sterile moths to one wild moth is needed. Male and female PBW both mate more than once, requiring the PBW-SIT program to maintain high ratios of sterile-to-native insects. This high ratio makes sterile insects impractical in heavily infested areas. The dynamics of the sterile insect itechnique strategy and its correlation to an insect's mating behavior is discussed by Davidson (1974). The sterile PBW moths are placed season long (average of 160 times per year) on approximately 25,000 hectares of the 350,000 hectares of cotton yearly in the San Joaquin Valley.

One of the premises of a program employing SIT is that the mass-reared and sterilized insects can compete successfully for mates with their native counterparts. Van Steenwyk et al. (1979) reported that mass-reared irradiated PBW males were less competitive than their native counterparts and that mass-reared and irradiated PBW females were equal to or more competitive than native females. However, they also indicated that the combined use of both male and female PBW provided a sterile population that was as competitive as native males and females in mating ability. The current PBW-SIT program uses both sexes. Miller et al. (1994)[a] reported that native and sterile PBW females were comparable in attracting and successfully mating with native males when confined in field stations. The authors also indicated that sterile male PBW entered commercial pheromone traps during the same time interval as native PBW males.

Miller et al. (1994)[b] indicated that native catches in the San Joaquin originated from possibly four sources. In descending order of likelihood they are: (1) Migrant populations from the heavily infested, more southern desert valleys of California; (2) Small native populations that do not reach detectable levels until late in the cotton growing season; (3) F_1 progeny developing from an out-cross of a native or migrant population and mass-reared moths; and (4) F_1 progeny from a self-cross of irradiated moths. The presence of the green fluorescent marker gene in PBW would provide a means to identify F_1 progeny from mating between the sterilized insects and natives. These "native" catches (F_1 progeny of sterilizedd insects) would not require increased uses of sterile moths because of the high degree of inherited sterility of such crosses (Miller et al. 1984). Current program policy requires that fields with native finds receive higher sterile insect use rates than those without native finds.

APPENDIX II

Pink Bollworm Integrated Pest Management

Cultural control:

The University of California Statewide Integrated Pest Management Project, Updated 12/98, at URL: http://www.ipm.ucdavis.edu/PMG/r114301511.html includes the following pink bollworm (PBW) integrated pest management (IPM): Eliminate the food supply for the PBW by cutting off irrigation early enough to stop production of green bolls by early September. Regardless of when the crop is terminated, immediately shred the cotton plants following harvest. Shredding destroys some larvae directly and promotes rapid drying of unharvested bolls. If fall temperatures are high during September and October, leave crop debris on the soil surface for two or more weeks after the shredding operation to further destroy larvae. Be sure to comply with plow-down requirements and cross-disc or plow to a depth of at least 6 inches (15 cm). Winter irrigations can reduce populations of overwintering PBW by as much as 50 to 70% and flooding in December is more effective than flooding in November or January. Take advantage of PBW mortality afforded by winter irrigations and rotate to small grains or newly seeded alfalfa. In spring, irrigations can also be used to promote early emergence of PBW. If cotton is being followed with cotton, pre-irrigate in February and plant as early as possible, following guidelines to ensure adequate soil temperature for germination and emergence. Plan irrigations of the crop to prevent even slight moisture stress and to promote maximum emergence of moths in advance of susceptible squares.

Pheromones , *Bacillus thuringinesis* Modified Cotton, Nematodes, Parasites:
The use of gossyplure, a sex pheromone attractant that disrupts mating when distributed throughout the field, may be effective against PBW when it is supplemented with cultural control practices (Staten et al. 1987). Genetically modified cotton that expresses *Bacillus thuringiensis* toxin is also effective to suppress PBW infestation and damage, since the PBW is one of the more susceptible cotton pests to the *Bt* proteinaceous toxin produced in the cotton plant (Flint et al. 1995). Three insect parasitic nematodes are of some interest for biological control of PBW (Henneberry et al. 1995). They are *Steinernema riobravis*, *S. carpocapsae* and *Heterorhabditis bacteriophora*. *Steinernema carpocapsae* has been available commercially, but costs are high and there have been product storage and transit viability problems in the past. Several species of exotic insect parasites have been introduced in California and Arizona for biological control of PBW, but none have become established, or have had any significant effect on pest suppression.

Table: Pesticides recommended for use in California Pink Bollworm IPM

Pesticide Name	Selectivity for PBW	Persistence on Natural Enemies
Gossyplure	High	None
Chlorpyrifos (OP)	Moderate	Short

Cypermethrin (SP)	Low	Moderate
Esfenvalerate (SP)	Low	Moderate

OP = organophosphate, SP = synthetic pyrethroid

Other insecticides that have been used against the pink bollworm include methamidophos (OP), encapsulated methyl parathion (OP), azinphos methyl (OP), methomyl (carbamate), permethrin (SP), and lambda-cyhalothrin (SP). Most of these pesticides are broad spectrum in toxic activity and can affect nontarget organisms, if exposed in sufficient quantities.

Monitoring:
Sampling bolls is the most reliable way to monitor high PBW populations. The University of California Statewide Integrated Pest Management Project, Second Edition of Integrated Pest Management for Cotton in the Western Region of the United States, 1996, Publication 3305 contains recommendations. See URL: http://169.237.210.130/IPMPROJECT/ADS/manual_cotton.html for availability. This publication provides detailed sampling methods and thresholds for control. Another University of California Statewide Integrated Pest Management Project, Phenology Model Database, provides a method of predicting PBW development that may be useful for timing insecticide applications and cultural control measures. See URL: http://www.ipm.ucdavis.edu/PHENOLOFY/pinkbollworm.html for availability.

APPENDIX III

Development of a Genetically Engineered Pink Bollworm

A. Transformation system:
The transformed pink bollworm (PBW) strain produced at the University of California, Riverside (UCR), originated from the mass-reared "C" stock of the Pink Bollworm Rearing Facility,

(PBWRF) in Phoenix, AZ. The origin of this PBWRF stock is from commercial cotton fields located in the Colorado River basin of California and Arizona. The PBW strains maintained in the PBWRF have been in existence since at least 1970. However, the colonies are periodically outcrossed with endemic field populations of PBW. The parental strain that was transformed was last outcrossed with wild-type PBW in 1996. All final engineering of the transforming constructs were performed at UCR. Of the transgenic PBW strains produced by UCR scientists (Peloquin et al. 2000), one strain (#35) was transferred to the APHIS, Plant Protection Laboratory in Phoenix, Arizona under USDA/APHIS permit No. 98-244-02m for movement of transformed insects between laboratories in Riverside and Phoenix.

The genes used from the donor organism and the *piggyBac*-derived portions of the vectors used to build the transforming construct were cloned off site. Specifically, *Escherichia coli* was the immediate host for the plasmids carrying the cloned genes used to make the transforming constructs. The *piggyBac* transposable element was discovered in cabbage looper cell culture at the University of Notre Dame (Fraser et al., 1995; Fraser et al., 1996; Wang and Fraser 1993). The *Bombyx mori* actin A3 promoter was cloned and modified by Thibault at the University of California, Riverside (UCR), using polymerase chain reaction (PCR) from the embryos of *Bombyx mori* purchased from Carolina Biological Supply Company.

B. Green fluorescent protein and *piggyBac:*

Green fluorescent protein and the ability of its derivatives to function as dominant, visible, nondestructive markers of insects (Brand 1995), mammalian (Pines 1995), and plant systems (Haseloff et al., 1997) were indicators of its potential use in PBW.

The Enhanced Green Fluorescent Protein (*EGFP*) gene is an enhanced version of the green fluorescent protein (*GFP*) gene cloned by Prasher, USDA, APHIS, Otis AFB, MA, from the jellyfish, *Aequora victoria* (Cubitt et al., 1995; Heim et al., 1994; Heim and Tsien, 1996; Prasher and Eckenrode, 1992, and Prasher, 1995). The plasmid source of *GFP* was purchased from Clontech, Inc. Previous plasmid-based mobility assays demonstrated that *piggyBac* or elements producing *piggyBac*-like transposase are not present, but when *piggyBac* is introduced as a donor/helper system, it is mobile in PBW embryos (Thibault et al., 1999). Therefore, a *piggyBac* vector was constructed containing EGFP as a marker for transformation.

The *piggyBac* element is a deoxyribonucleic acid (DNA) transposable element capable of integrating into other DNA through mediation of a transposase encoded by a transposase open reading frame (ORF) within the element, only when its inverted terminal repeats (ITR) are intact. In the construct used for transformation of the PBW, the transposase gene of the *piggyBac* element was destroyed by insertion of an expression cassette containing *EGFP* ORF driven by a single copy of the *Bombyx mori*-derived BmA3 promoter. This manipulation destroys the ability of the transformation construct to move on its own. Transformation was done by co-injecting a transposition and integration incompetent helper plasmid along with a donor plasmid into early stage PBW embryos. The donor plasmid contains the transforming construct flanked by *piggyBac* ITRs. The helper plasmid encodes an intact *piggyBac* transposase ORF. The gene product of this *piggyBac* transposase ORF is under the control of a promoter that directs insect cells to express *piggyBac* transposase after injection. Importantly, the helper plasmid lacks the downstream *piggyBac* ITR. These ITRs are absolutely essential for *piggyBac* transposase mediated integration. Therefore, the helper plasmid, lacking one or the other of the ITRs, cannot

integrate itself into target DNA in a transposase-mediated event.

The potential for instability and unwanted mobilization of *piggyBac*-derived transforming constructs is addressed in the following: Although there is no evidence for any *piggyBac* transposase activity in the PBW genom, it could be argued that, if there were endogenous *piggyBac*-like elements undetected in the applicants screen for these elements in PBW, they might provide a source of transposases that could mobilize *EGFP* transgenes flanked by *piggyBac*-derived ITRs. Demonstration of elements homologous to *piggyBac* in the recipient PBW might then imply some instability of the *EGFP* transgene. However, there is no evidence for *piggyBac*-like transposases in PBW (Thibault et al., 1999). Additionally, the DNA-mediated element, *Hermes*, has been used to successfully transform *Aedes aegypti* with little or no evidence of instability of the transgenes over at least 10 generations, even though there are endogenous elements (hAt-like, as is *Hermes*) in *Aedes aegypti* with close enough homology to *Hermes*, so that these endogenous hAt and *Hermes*-like elements are detected in higher stringency Southern blots with a *Hermes* probe (Jasinskiene et al 1998). In the case of pink bollworm, low stringency Southern blot experiments on pink bollworm DNA with radiolabeled DNA probes derived from *piggyBac,* which would be even more likely to detect elements with low homology to *piggyBac* than the higher stringency methods used by Jasinskiene, et al., were unable to detect any endogenous *piggyBac*-like elements. This indicates that there are no elements in the PBW that might reasonably be expected to mobilize a *piggyBac*-derived transgene. In addition, excision and transposition assays were performed in PBW embryos with *piggyBac*. This was primarily to determine if *piggyBac* could integrate into the PBW genome. However, results of transposition assays did not show transposition of *piggyBac* in the absence of exogenous *piggyBac* transposes, indicating there were no unknown *piggyBac*-like elements in the PBW genome (Thibault et. al. 1999). Thus, there should not be unexpected interactions between the components of the PBW genome and the transforming construct that could result in instability of the transgenes.

C. Molecular characterization of engineered pink bollworms:
Insertion of the *piggyBac* element into genomic DNA was detected by Southern blot analysis using one of the positive lines. The presence of at least two insertions was detected in this line with the probe recognizing approximate 1.9-kb and a 2.3-kb bands. Individuals examined contained either one of the inserts, or both. Based on inverse PCR, the *piggyBac* integration appears to have been a singular event which occurred in a transposase-dependent manner resulting in a target site duplication with no plasmid sequences flanking the transposon ends. Immunoblot analysis using a green fluorescent protein-specific antibody was also used to differentiate expression of EGFP from autofluorescence in wild-type animals and establish that the EGFP protein produced was the expected size showing that no additional sequence was being translated into protein fused to the EGFP.

The helper plasmid contained a *piggyBac* transposase gene driven by the *Drosophilia hsp70* heat-shock promoter instead of the endogenous *piggyBac* promoter that was obliterated by introduction of the *Drosophilia hsp70* promoter. Integration of the *Bombyx mori A3 EGFP* construct, which lacks sequences found in the original element, also demonstrates that the whole *piggyBac* element is not essential for transposase-mediated transposition. The complete element is 2.5 kb in length. Construction of the vector resulted in deletion of approximately 1 kb within the original *piggyBac* transposase open reading frame, resulting in inactivation.

D. Stability of genetic integration

The enhanced green fluorescent protein (EFGP) positive lines were maintained as heterozygotes by serial backcrosses to the wild-type strain. At the time of backcross analysis, the lines had been backcrossed for four generations. This would likely separate any transformed loci that were not tightly linked. Thus, the EGFP-positive parental insects used in the diagnostic backcrosses were expected to be heterozygous for a single copy of the gene. At the time of backcross analysis of the heterozygote lines, the first line produced 191 positive and 207 negative progeny and the second line produced 555 positive and 616 negative progeny. These were not significantly different from the expected 1:1 ratio by χ^2 statistical analysis. Therefore, a relatively close 1:1 ratio of EGFP versus wild-type supports the hypothesis that *EGFP* was transmitted as a single-locus, dominant gene.

E. Fitness compared to wild-type

The stability of the gene was demonstrated further by the rearing at the Phoenix Quarantine Facility of 20 generations of EGFP strain PBW with no evidence of change of the *EGFP* gene. This study found no differences in length of time spent in larval instars, and the pupal stage in EGFP PBW compared to non-transformed PBW. However, the EGFP female moths produced 19.8 % fewer eggs than non-transformed PBW and their successfrul egg hatch rate was 26% lower (Miller et al., 2001).

APPENDIX IV

Description of Transgenic Pink Bollworm Fitness Tests to be Performed in Field Cages

TEST 1—This test will compare male response to pheromone traps of EGFP strain and non-EGFP strain PBW. This test will be replicated six times using two cages with 2-week intervals between experiments using 50 males per replicate per treatment. All EGFP strain males will be marked externally with a pink florescent dye. Control (non-EGFP) insects will be marked with a blue florescent dye. Two Delta™ traps baited with 2 mg of gossyplure PBW pheromone impregnated in a rubber septum will be placed in each cage 24 h after the moths are put in the cages. Traps will be examined daily for moth captures. All moths will be sterilized with a radiation dose of 20–kr of $Cobalt_{60}$ for an additional measure of biological confinement.

TEST 2—This test compares male longevity in the field of EGFP and non-EGFP strain PBW. Each of three field cages will receive 100 EGFP strain PBW males and 100 non-EGFP strain males. Each PBW strain will be marked with a different color fluorescent dye as described in Test 1. Following moth placement in cages, one of the three cages will be randomly assigned to have two Delta traps baited with 2 mg of gossyplure placed in the cage five days after the moths are put into the cage. Of the remaining two cages, one will have two traps placed in the cage on day-10 after moths are put into the cage and the remaining cage will have the two traps placed in the cage on day-15 after moths are put into the cage. The test will be replicated five times using 300 males per treatment, per replicate. All moths will be sterilized with a radiation dose of 20–kr of $Cobalt_{60}$ for an additional measure of biological confinement.

TEST 3—This test compares the EGFP and non-EGFP females' ability to solicit and mate with their male counterparts and males from the different strain. Each female moth will have the wings on the left side surgically removed while under cold anesthesia in the laboratory. Pairs of females, consisting of one EGFP and one non-EGFP strain, will then be placed for 24 h in a 9-dram vial fitted with a 0.5-dram feeder vial containing a 6% sucrose solution. These vials will be used to transport the moths to the field mating stations located in the screen field cages. Pairs of female moths will be placed in each of ten mating stations at dusk along with a cotton leaf and exposed to 100 males. At dawn on the following morning, the females will be removed from the stations and returned to the laboratory. Each female will then be dissected to determine mating by the presence of a spermatophore in the bursa copulatrix. If mating has occurred, the type of male she mated with will be determined. Males from the non-EGFP strain will be mass-reared on a larval diet that contains a fat-soluble dye that internally marks the insect. The dye is also retained in the spermatophore that the male transfers to the female. Thus, the spermatophore from non-EGFP strain males can be differentiated from those of the EGFP strain when examined under a microscope. All moths will be sterilized with a radiation dose of 20–kr of $Cobalt_{60}$ for an additional measure of biological confinement.

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